PREVENTION AND TREATMENT OF MILITARY IMPORTANT DISEASES IN THE TROPICS

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1. Adenosine Deaminase in Human Malaria Infection

PROBLEM : Hereditary deficiency of the purine enzyme, adenosine deaminase is associated with severe combined immunodeficiency disease (SCID) - a condition in which both T- and B-lymphocyte function is impaired. Partial restoration of lymphocyte function can be achieved in SCID patients by enzyme replacement therapy involving whole blood or packed RBC transfusion. It thus appears that the ADA in normal RBC is sufficient to correct in part the purinogenic defect in ADA deficient lymphocytes. The precise mechanism for this effect is not understood although it may involve the role of the red cell mass in systemic adenosine metabolism in a way that influences purine metabolism in lymphocytes. Acute human malaria infection is characterized by immune suppression. In particular there is a decreased functional responsiveness of mononuclear cells - especially T-lymphocytes. It is also known that the intraerythrocytic malaria parasite produces major changes in the purine metabolism of the host red cell mass.

PROGRESS : We have studied purine metabolism in erythrocytes and lymphocytes from Thai adults naturally infected with *P. falciparum*. Heparinized whole blood was used to prepare lysates for enzyme studies and perchloric acid extracts for purine nucleotide profiles. Adenosine deaminase (ADA) was measured by a radiochemical method involving an automated HPLC system. Nucleotide profiles were determined by an anion-exchange gradient HPLC method. Adenosine deaminase activity was increased in *P. falciparum* infected erythrocytes from individuals with naturally acquired infection. Nucleotide profiles on individuals with low parasitemias (0.5 percent) showed an increased in erythrocyte adenosine triphosphate (ATP) levels. Adenosine which is not deaminated by ADA would be available for phosphorylation to ATP by adenosine dinase. Nucleotide profiles on lymphocytes revealed a decrease in ATP levels. This suggest that the energy potential of lymphocytes in malaria infected individuals is reduced and that the lymphocytes' ability to respond metabolically is depressed.

FUTURE OBJECTIVES : These studies suggest what may be a biochemical correlate of immune dysfunction in human malaria infection. The perturbation in

erythrocyte adenosine metabolism caused by the malaria parasite may produce a defect in lymphocyte purine metabolism which renders this cell functionally defective. Work is currently underway to confirm these preliminary observations in a larger population study. Specific emphasis is being given to studies on malaria lymphocytes adenosine metabolism, adenosine receptors and cyclic nucleotides.

2. Adenosine Deaminase in Malaria Infections : Effect of 2'-Deoxycoformycin *in vivo*

PROBLEM : Purine nucleotides are required by the rapidly proliferating malaria parasite for both energy metabolism and nucleic acid synthesis. The malaria parasite cannot synthesize purines *de vono* and depends for its intraerythrocytic (IE) growth and development on salvage of purine bases from the host RBC and extracellular environment. We have shown with *Plasmodium falciparum, in vitro,* that hyposanthine is utilized as a purine base precursor for parasite synthesis of adenosine and guanosine nucleotides and that specific inhibition of these synthetic pathways leads to parasite destruction. Whether hypoxanthine is the malaria parasites preferred substrate *in vivo* is not known. An increase in adenosine deaminase (ADA) activity, however, is an obvious means for production of IE hypoxanthine. Increased availability of hypoxanthine would be a natural consequence of adenosine metabolism in the mature erythrocyte (viz : $\xrightarrow{ADA}$ inosine $\xrightarrow{PNP}$ hypoxanthine) since this cell lacks the enzyme xanthine oxidase. Conversely, inhibition of ADA activity could act to deprive the rapidly growing IE malaria parasite of a readily accessible hypoxanthine pool for purine nucleotide synthesis. It was, therefore, of interest to determine whether specific inhibition of ADA activity *in vivo* using the tight binding inhibitor, 2'-deoxycoformycin, interferred with the malaria parasites IE growth and development.

PROGRESS : Adult male rhesus monkeys with no previous exposure to malaria infection were experimentally infected with *Plasmodium knowlesi.* Samples of whole blood were collected at selected times during the IE infection cycle and at various levels of parasitemia. Lysates were prepared from washed RBC. The ADA assay was done by a radiochemical HPLC methods which measured the conversion of (^{14}C) adenosine to (^{14}C) inosine. There was a 3.6 fold increase in RBC ADA activity of infected rhesus with a mean parasitemia of 6.2 percent parasitized RBC (1.91 ± 0.18 vs 6.95 ± 0.70) nanomoles/min/mg protein, control vs infected RBC; n = 12). ADA levels were observed to increase with increasing parasitemias in serially sampled animals. Malaria parasitized RBC (PRBC) have been shown by starch gel electrophoresis to contain a distinct parasite ADA enzyme. It is apparent, therefore, that IE malaria parasite growth and proliferation is associated with an increase in PRBC ADA activity. 2'-Deoxycoformycin (DCF) (single i.v. dose, 250 mg) effectively inhibited RBC ADA activity in malaria infected monkeys at six and 24 hours. Parasitemias were decreased at six hours and continued to fall over the ensuring 24 hours. Microscopic examination of six hour DCF treated PRBC revealed parasite nuclear and tytoplasmic deterioration. By 24 hours the majority of PRBC contained degenerate parasite forms. DCF thus appears to produce a potent antimalarial effect *in vivo*.

FUTURE OBJECTIVES : Adenosine deaminase appears to be a potential metabolic target for the design of new anti-malarial chemotherapy. Work is currently underway to more fully understand the action of 2'-deoxycoformycin on the malaria parasite and the role of ADA in parasite development and proliferation.

3. Antibody Secretions of Malarious Individuals (Immuregulation in Malaria)

PROBLEM : To examine the synthesis and secretion of total IgM, IgG and IgA during 12 days of *in vitro* culture, by peripheral blood mononuclear cells from malarious Thais.

PROGRESS : The presence of autoantibodies is often taken as an indication of an alteration in immunoregulation. Studies on the nature of antibody synthesis and secretion by MNC from malarious patients are of importance because of the ability of *in vitro* antibody production to serve as a uniquely sensitive indication system for studying immunoregulatory pathways as well as delineating functional and qualitative alterations in both the humoral and cellular components of the immune response during malarial infection.

We utilized a solid phase system, with commercially obtained rabbit anti-IgA, IgM or IgG covalently bound to a cross linked polyacrylamide bead. Specificity was tested with purified immunoglobulins with no cross reactivity detected. The IgG, IgA, and IgM synthesized and secreted into the media were measured by separate solid phase radioimmunoassays for each immunoglobulin class with specific rabbit anti-immunoassays and 125I-labelled immunoglobulins. The assays were performed in 96 well, round bottomed, microtiter plates. To each well was added 10u of culture supernatant or standard, 50u of radio labelled IgG, IgA, or IgM (25,000 CPM), and 50u of appropriate solid phase antisera. After overnight incubation at 25°C, the wells were resuspended and harvested on glass fiber filter strips using a Bellco microharvester. Individual discs were counted using a gamma counter. Presently, we are standardizing the antibody synthesis assay.

FUTURE OBJECTIVES : Studies on the immunoregulation to malarial infection are of importance. Increasing resistance to the parasite and the developmental work on malarial vaccine emphasizes the importance of these studies.

4. Subpopulations of T Cells (Tg and Tm) in Patients with Malaria

PROBLEM : To quantitate subpopulations of T cells (Tg suppressor and Tm Helpers) in the peripheral blood of patients with malaria.

PROGRESS : In the present study we utilized rosetting techniques to enumerate the putative suppressor (Tg) and helper (Tm) T-cell subpopulations in the peripheral blood of adult Thais with malaria. A lower percentage of both Tg and Tm subpopulations and a lower number and percentage of total T cells was found in these patients during the acute period of infection than in the peripheral blood of healthy donors. However, the percentages of total T, Tg and Tm cells were higher during the convalescent period and were comparable to

the value found in the peripheral blood of healthy donors. No correlations were found between the percentages of these T-cell subpopulations and the level of parasitemia or the hematocrit.

FUTURE OBJECTIVES : Further studies should be conducted to assess the sequential development of the host (human and primate) immune responses to malaria infection.

5. Examination of Sera from Indonesians with Malaria Splenomegaly Syndrome in Assays of Blastogenic Responsiveness to Mitogenic Lectins and Cell Surface Antigens

PROBLEM : To examine the effect of sera from Indonesians with Malarial Splenomegaly Syndrome on the cellular immune function of human mononuclear cells using the mitogen induced lymphocyte transformation assay and the mixed leukocyte culture system.

BACKGROUND : We have previously shown that the mitogenic responsiveness of normal peripheral blood mononuclear cells was markedly reduced to both PHA and Con A when 20 percent pooled or individual sera from patients with *P. falciparum* and *P. vivax* malaria were added to the mononuclear cells. Sera from patients also displayed an inhibitory effect on the normal bastogenic response to allogeneic cell surface antigens *in vitro*. In a collaborative study with the NAMRU 2 laboratory we are examining sera from individuals with tropical splenomegaly syndrome in an effort to delineate whether inhibitory characteristic are present and can be associated with clinical findings.

METHODS : Methods have been previously described in detailed (2).

6. The Effect of Anti-coagulants on Cold Reactive Anti-lymphocyte Activity in the Blood of Patients Naturally Infected with Malaria

PROBLEM : To compare lymphocytotoxicity in malarious patients plasma and serum.

PROGRESS : The effect of three different anti-coagulants on the level of cold-reactive anti-lymphocyte activity (ALA) in the peripheral blood (PB) of malarious individuals was assessed to determine if plasma could be substituted for serum in assays designed to characterize ALA. Plasma was obtained from PB previously treated with Heparin, acid-citrate dextrose (ACD), or ethylenediamine tetraacetic acid (EDTA). An equivalent level of ALA was found in the serum and plasma obtained from ACD or EDTA treated blood, however, ALA in the Heparin treated blood was substantially lower. Thus, it appears that plasma obtained by treating PB with ACD or EDTA, but not heparin can be used instead of serum to investigate the role of anti-lymphocyte factors in malarial infections. The major practical advantage of this procedure is the higher yield of MNC and plasma to investigate the interactions of lymphocototoxic factors and autologous MNC.

FUTURE OBJECTIVES : This study is complete.

7. Kinetics of Japanese encephalitis (IgM and IgG Human Serum and CSF

PROBLEM : Japanese encephalitis (JE) is endemic in Southeast Asia and has a high case fatality rate. Existing methods of diagnosis (Hemagglutination inhibition-HAI) are adequate for retrospective studies but results cannot be obtained early enough to affect treatment of the disease.

PROGRESS : Thirty-two patients with a clinical diagnosis of encephalitis have been studied during the 1981 epidemic season in the provincial hospital at Kampangphet, a province with a high rate of JE. JE could be quickly diagnosed by the demonstration of specific anti-JE IgM in the CSF by IgM antibody capture (MAC) immunoassays. CSF samples from 25 patients with other diseases with possible CNS involvement were negative of JE IgM.

Five siblings of encephalitis cases with demonstrable asymptomatic JE had JE IgM in their serum but not in their CSF.

FUTURE OBJECTIVES :

1. JE MAC immunoassays should replace conventional HAI serology for diagnosis of JE.

2. JE MAC immunoassays should be used to select patients for trial of promising antiviral drugs.

8. Production of Flavivirus Temperature Sensitive Mutants

PROBLEM : The existence of a battery of temperature sensitive (ts) mutants of flaviviruses and the demonstration of complementation would allow investigation of the biochemical functions of nonstructural virus specified proteins, the identification and characterization of the lesion in candidate vaccine viruses and in the field isolates, and determination of the relationship of protein function to virus virulence.

PROGRESS : The heat resistant strain of Japanese encephalitis virus (JEV) has been treated with two additional mutagens N-methyl-N'-Nitro-N'-Nitrosoguannidine (NG) and Fluorouracil (FU). Virus treated with NG has been cloned and 422 clones have been tested for ts character. Of these clones eight were stable ts mutants. Virus grown in the presence of FU has been cloned and 248 clones have been tested for ts character. Of these clones nine were stable ts mutants.

FUTURE OBJECTIVES :

1. These stable mutants are being characterized as to mutant function and should be analyzed for complementation with other mutants.

2. If complementation can be demonstrated, field isolates of JEV should be tested to determine the frequency of naturally occurring ts mutants, the nature of their lesion and its relationship to pathogenesis.

9. A Primate Model for Hemorrhagic Dengue

PROBLEM : To attempt to develop an animal model for DHF to determine the role of enhancing maternal antibody.

PROGRESS : We collected dengue type 2 virus isolates from Thai children less than one year old hospitalized in Bangkok with hemorrhagic fever and also collected serum specimens from the mothers of these infants. Mothers' sera were screened for their ability to enhance growth of the corresponding infant's virus *in vitro* in cultures of a continuous mouse macrophage cell line. One maternal serum-infant virus pair which showed strong *in vitro* antibody dependent enhancement (ADE) of virus growth was selected for use in primate inoculations. The virus chosen had been isolated from a fatal case of DHF. The "DHF maternal sera" selected produced maximal ADE of virus growth *in vitro* at a 1:1000 dilution.

Colony born infant primates, ages eight to 18 months, were the study subjects. Clinical signs were monitored, and bloods were drawn daily for measurement of hematocrit, white blood cells, platelets, viremia, and antibody response. All monkeys were inoculated with virus strain intravenously. Experimental monkeys received "DHF maternal sera" intravenously 24 hours prior to virus injection while control monkeys received similar injections of non-immune human sera.

Six infant cynomolgus monkeys were inoculated with serum doses calculated to give a 1:3000 or 1:10,000 dilution of the "DHF maternal serum" in the primate's extravellular fluid (ECF) space. No disease was observed, although both the viremia and antibody response occurred earlier in the monkeys that received the "DHF maternal serum".

Four infant rhesus monkeys were inoculated with serum doses calculated to give a 1:500, 1:1000, 1:2000, or 1:5000 dilution in the ECF space. The monkeys pretreated with the two highest doses of "DHF maternal serum" developed profound (< 30,000 platelets per mm^3) thrombocytopenia five to 10 days after virus inoculation. The monkey receiving the 1:1000 serum dose developed a positive tourniquet test, multiple spontaneous bleeding sites, and died of hemorrhagic shock. The two monkeys receiving lower doses of "DHF maternal serum" and all four monkeys which received pretreatment with normal non-immune sera all remained well without thrombocytopenia.

FUTURE OBJECTIVES : Early results indicate a feasible model. Further work should be done studying the parameters of the model.

10. Ectoparasite and *Rickettsia tsutsugamushi* Studies in Thailand

PROBLEM : The goals of this research are to establish and describe ectoparasites that are or are potential vectors of human parasites or pathogens of human disease in Thailand, and to delineate the distribution of natural populations of larval mites infected with *Rickettsia tsutsugamushi* in Thailand.

PROGRESS : Collaborative studies between the Department of Medical Entomology, AFRIMS, and the USAMRU, Kuala Lumpur, Malaysia, have shown that several strains of *Rickettsia tsutsugamushi* occur in nine different mite species in various parts of Thailand (3). Some of these species are new and are being or have been described (4).

Collections of ectoparasites were made from rodents collected in attempts to isolate Hantann virus in the port area of Bangkok. Several species of lice, ticks, fleas, and mite were found to heavily infest the rats occurring in the vicinity of the warehouses and foodstalls. Hantann virus was isolated from a rat captured near a large foodstall.

After some revisions The Checklist of the Ticks of Thailand has been accepted for publication is currently in press (5).

FUTURE OBJECTIVES : With the discovery and identification of at least four new species of chiggers that were found to contain *Rickettsia*, future collaboration with the laboratory in Malaysia is planned in order to determine the role these mites play in human disease transmission. Temporal and spatial relationships between scrub typhus and its vectors will be investigated where human cases have been contracted.

11. Leptospirosis in the Non-Human Primate Model : Chemoprophylaxis and Early Diagnosis of Infection.

PROBLEM : Leptospirosis is a common zoonotic disease found throughout the world. The clinical features in man range from an influenza-like illness to a more severe disease form manifested by continued fever with meningitic symptoms and signs (6,7). In some cases infection can lead to renal and hepatic failure, jaundice, and even death (6,8). Leptospirosis is frequently found in the tropical areas of the world (9,10) and recent attention has focused on several outbreaks in soldiers training in jungle areas (11). Symptomatic treatment and antibiotic therapy is used in the acute illness. However, once symptoms are evident the beneficial effect of antibiotics is questionable (7). The relatively long recovery period, even with treatment, suggest that prevention is the practical approach in solving the problem of leptospirosis. It is difficult to prevent direct contact with leptospira contaminated water in a tropical environment, especially during military maneuvers. Immunization against specific serovars of leptospira can protect animals but immunization of man is not practical unless the serovar endemic to the area is identified or a vaccine with broad antiserovar activity is developed.

The current objectives are:

1. To characterize clinical leptospirosis in the non-human primate model.

2. To determine the efficacy of antibiotic treatment as a disease prophylaxis for the acute infection.

3. To determine if an ELISA method for detecting leptospira antibody or antigenemia is a useful means for obtaining rapid early diagnosis of leptospirosis.

PROGRESS : A pilot study has been completed in *Macaca mulatta* and *Macaca irus* monkeys infected with a human isolate of *Leptospira bataviae*, a strain commonly isolated from patients in Thailand with clinical leptospirosis. Following intraperitoneal injection of 10^7 organisms, a leptospiremia was detected in four of five *M. irus* and four of four *M. mulatta* for a period of one to six days after infection. Some CSF cultures were also positive for *Leptospira*. A febrile response was present in these monkeys on days two, three, and four. All monkeys survived the infection. Serum and cerebrospinal fluid samples were obtained for future testing for antibody and leptospiral antigen.

FUTURE OBJECTIVES :

1. Doxycycline, given daily as a prophylactic measure will be tested in monkeys given *Leptospira bataveae*.

2. If the antibiotic prophylaxis is successful in preventing bacteremia and fever, this treatment will also be tested in the weaning hamster infected with *Leptospira*. This infection in hamster is usually lethal. The minimum inhibitory concentration of doxycycline for *Leptospira in vitro* will also be determined.

11. The Diagnosis of Canine Rabies Infection Using the "Antibody Capture" Solid Phase Elisa Method (Acelisa)

PROBLEM : Rabies is endemic in Thailand. In a recent report over a ten year period, the yearly range was 237-322 cases in man and 871-3286 cases in dog (12). With such a high incidence of disease, a reliable, rapid method of early diagnosis of infection is important. Many laboratory techniques have been developed for the diagnosis of rabies. The Seller's stain for Negri bodies, the Fluorescent Antibody technique (13), the radio immunoassay (14) and detection of IgM after vaccination by immunoperoxidase method have all been used for the diagnosis of rabies infection in man and animals. Each of the tests have required either considerable time or sophisticated equipment to obtain the result. The detection of low level (IgM) in human serum has been reported at the 3rd and 4th day post exposure to rabies antigen (15).

The antibody capture solid phase enzyme linked immunosorbent assay (ACELISA) has one of a unique properties that is gross specific immunoglobulins are functionally concentrated onto the solid from the liquid so that very low concentration can be detected (16). We propose to use this method to detect the acute rabies infection in the dog by measuring the level of IgM in CSF as well as in serum. This method may provide a means for early diagnosis of the canine rabies infection.

The current objectives are :

1. To determine the onset of detectable IgM in the CSF and sera of dogs with acute rabies infection.

2. To determine whether the rabies virus is shed into the CSF sera during an acute phase of illness.

3. To develop a simple, rapid, reliable technique for the diagnosis of canine rabies infection that does not require long quarantine or killing of the dog for testing.

4. To compare the result of the ACELISA technique with other established methods for the diagnosis of acute rabies infection.

PROGRESS : Serum and CSF were collected from 18 rabid and 29 non-rabid dogs. All serum and CSF will be tested for rabies antibody titer by ACELISA and REFIT.

13. Antibody Capture RIA for Diagnosis of Rabies

PROBLEM : Previous work has demonstrated that Japanese B cencephalitis can be reliably diagnosed early in an illness by determining the specific activity of anti-JE IgM in CSF and serum. The concept that locally synthesized specific antiviral IgM in the CSF might allow diagnosis of other forms of viral encephalitis required further evaluation. The presence of a privately supported study of the clinical management of rabies encephalitis provided the opportunity to study sera and CSF from several well-diagnosed local cases of encephalitis. Ultimately, this study will describe the kinetics of IgG and IgM anti-rabies antibody in serum and CSF in a manner similar to that used for Japanese encephalitis.

PROGRESS : During the summer of 1982, sera from five patients receiving Merieux Institute B-propionolactone inactivated vaccine were used as pilot specimens. An IgM capture (MAC) RIA was designed. Rabbit anti-human IgM was bound to the plates, the serum specimen washed over it, Merieux vaccine antigen applied, and finally 125_I tagged, purified IgG from a rabies hyperimmunized rabbit added. The Merieux vaccine was superior to rabies infected neuroblastoma or BHK cells. The optimum dilution of antigen was a 1/4 dilution of vaccine. Sera from 14 days post-immunization bound 2.7 times more tagged antibody than pre-immunization sera. Post vaccination sera were clearly distinguishable from pre-vaccination sera when tested for IgM. The IgG antibody capture (GAC) assay is presently under development.

FUTURE OBJECTIVES :

1. Continue to determine optimum conditions for rabies MAC and GAC.

2. Proceed to test sera and CSF from patients with diagnosed rabies.

3. Ascertain the kinetics of anti-rabies antibody production in serum and CSF, especially in relationship to pathogenesis of rabies encephalitis.

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